Vol. LXIV, No. 3 June, 1933

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

HEMOLYTIC ACTION OF SILVER OCCURRING AS AN IMPURITY IN CHEMICALLY PURE SODIUM CHLORIDE [1]

ERIC G. BALL [2]

(From the Marine Biological Laboratory, Woods Hole, Mass., and Department of Physiological Chemistry, Johns Hopkins Medical School)

The hemolysis of fish erythrocytes when placed in isotonic solutions made from certain brands of sodium chloride C.P. has been reported by Williams and Jacobs (1931). The experimental results presented by these investigators suggest an impurity in the salt as the causative agent. Since sodium chloride is the main constituent of most isotonic mediums used in biological experiments it seemed of importance to determine the exact nature of this toxic principle. Data will be presented in this paper to show that the hemolysis of fish erythrocytes by isotonic solutions made from certain brands of so-called chemically pure sodium chloride is due to the presence of small amounts of silver occurring as an impurity in the salt.

Procedure

Fish were bled directly from the ventral aorta into a beaker. The blood was defibrinated and the fibrin removed by filtering through dry cheesecloth that had been washed in distilled water. The blood was then centrifuged for five minutes at a rate just sufficient to cause separation of the cells and serum and the cell volume roughly adjusted when necessary to the normal value of 25 per cent so as to insure uniformity in the cell content of blood obtained from different fish. This procedure was found to be necessary as the rate of hemolysis was affected by the amount of red cells used. One drop of the remixed blood was added from a bulb pipette, constructed so as to deliver uniform drops (1 cc. = 28 drops), to 25 ml. of the isotonic (0.25 M) sodium chloride solutions contained in test tubes (200 × 30 mm.). The progress of hemolysis with time was followed by visual inspection.

[1] Presented before the American Society of Biological Chemists at Philadelphia, April 28–30, 1932.

[2] With the coöperation of Harold Blumberg, Department of Biochemistry, Johns Hopkins School of Hygiene and Public Health.

Results

The blood cells of different species of fish show a wide variation in their resistance to hemolysis when placed in isotonic solutions of various brands of sodium chloride. In Table I are assembled representative times for complete hemolysis of the blood cells of a number of species of marine fish when placed in isotonic solutions made from four different brands of sodium chloride. The blood cells of both the

Table I

Hemolysis time for the erythrocytes of various species in isotonic sodium chloride

Species	Brand 1	Brand 2	Brand 3	Brand 6
Cunner (*Tautogolabrus adspersus*)	No hemolysis	5 min.	6 min.	7 min.
Tinker Mackerel (*Scomber scombrus*)	No hemolysis	5.5 min.	7.5 min.	27 min.
Bull's Eye Mackerel (*Scomber colias*)	No hemolysis	3 min.	4 min.	5 min.
Butterfish (*Poronotus triacanthus*)	No hemolysis	4 min.	5 min.	10 min.
Flounder (*Platessa dentatus*)	No hemolysis	10 min.	10 min.	15 min.
Scup (*Stenotomus chrysops*)	No hemolysis	18 min.	32 min.	
Tautog (*Tautoga onitis*)	No hemolysis	22 min.	34 min.	
Common Sea Robin (*Prionotus carolinus*)	No hemolysis	25 min.	25 min.	30 min.
Rudder Fish (*Palinurichthys perciformis*)	No hemolysis	25 min.	25 min.	20 min.
Dogfish (*Mustelus canis*)	No hemolysis	75 min.	60 min.	

mackerel and the cunner are rapidly hemolyzed. Since both of these species were easily obtainable and afforded a rapid means of testing for the toxic principle they were used throughout in the work that follows. (Table VI excepted.)

Of the four brands shown in Table I only No. 1 was non-toxic. This is a Kahlbaum's salt, which agrees with the findings of Williams and Jacobs. This salt is the only one which is not mined. It is prepared from sodium carbonate and hydrochloric acid in porcelain vats. This fact would immediately lead one to suspect the occurrence of an

impurity in the other salts. However, when seeking the cause for toxic actions of single salt solutions on biological material it is always necessary to keep in mind the action of so-called antagonistic ions. That the lack of antagonistic ions cannot be the explanation in this case is shown by the results presented in Table II.

An isotonic solution of sodium chloride which showed rapid hemolysis was progressively diluted with sea water that had also been made isotonic by dilution and which caused no hemolysis. Since sea water contains a suitable mixture of the ions deemed necessary to form a physiologically balanced solution, such admixture should result in the

TABLE II

Effect of dilution of toxic salt solution with isotonic sea water.
0 = no hemolysis; ++++ = complete hemolysis.

Isotonic* sea water	0 ml.	5 ml.	10 ml.	15 ml.	20 ml.	25 ml.
NaCl No. 2	25 ml.	20 ml.	15 ml.	10 ml.	5 ml.	0 ml.
Hemolysis Time *min.*						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	++	+	0	0	0	0
5	++++	+	0	0	0	0
6		++	+	0	0	0
7		++++	++	0	0	0
8			++	0	0	0
9			+++	+	0	0
10			++++	++	0	0
15				++++	0	0
100					++++	0

* Similar results obtained when a non-toxic salt solution substituted for isotonic sea water.

complete disappearance of the toxic action. However, as can be seen from the data presented, the toxic effect persisted, though diminished, even when sea water formed as much as 80 per cent of the mixture. Moreover, almost identical results were obtained when the dilution was made with a non-toxic sodium chloride solution. Thus the presence of calcium, magnesium, and potassium had no effect on the toxic action. Therefore, it seemed reasonable to conclude that the toxic action was caused by an impurity.

In order to determine whether this impurity present in sodium chloride was organic or inorganic, samples of salts that showed hemolytic effects were fused in a platinum crucible and held in the liquid

state for at least fifteen minutes. Such treatment should destroy, at least partially, any organic matter present. Hence if the impurity is organic in nature, isotonic solutions made from the fused salt should cause little or no hemolysis whereas the toxic action should remain undiminished if the impurity is inorganic.

As shown in Table III, fusing caused no diminution in the toxic action of a salt; in fact, an increase in toxicity seemed to have occurred. Hence the impurity was classed as an inorganic substance. The only reasonable explanation for the increased toxicity after fusion seemed to be the formation of oxides with an attendant increase in alkalinity of the resulting solution. Rough tests with indicators showed that

TABLE III

Hemolytic action of various salts before and after fusion.

S.G. = swirl gone—disappearance of characteristic swirl shown by cells on shaking.

Salt*	1	1 Fused	2	2 Fused	3	3 Fused	4	5	6
Hemolysis Time *min.*									
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	S.G.	++	0	++	0	0	0
4	0	0	++	++++	S.G.	++++	0	0	0
5	0	0	++++		S.G.		0	0	S.G.
6	0	0			+		0	0	+
7	0	0			++		0	0	+++
8	0	0			+++		0	0	++++
9	0	0			++++		0	0	
10	0	0					0	0	
300	0	0					0	0	

* Each number represents a different brand of sodium chloride, except No. 5 which was prepared from recrystallized sodium carbonate and hydrochloric acid.

such was the case, suggesting that the toxic action might vary with pH.

In the hope that some light might be thrown on the nature of the inorganic impurity present in the sodium chloride, a study of the effect of pH on the rate of hemolysis was made. The results of one such experiment are shown in Table IV. The pH was controlled by adding 2 ml. of buffer to 23 ml. of isotonic salt solution, a procedure which altered the isotonicity but little. The difference in pH produced by dilution of the buffer was not detectable colorimetrically. As the pH increases, the rate of hemolysis also increases. Below pH 5.0 the hemolytic action is very slow and if present is masked by a change produced on the cells by the acid solutions. This effect of pH sug-

TABLE IV

Effect of pH on Hemolysis Time.

pH	3.0		4.0		5.0		6.0		7.0		8.0		9.0		10.0	
Salt*	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Hemolysis Time *min.*																
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0	++
3	0	0	0	0	0	0	0	0	0	+	0	+++	0	++++	0	++++
4	0	0	0	0	0	0	0	0	0	+++	0	++++	0		0	
5	0	0	0	0	0	0	0	+	0	++++	0		0		0	
10	0	0	0	0	0	+	0	++++	0		0		0		0	
15	0	0	0	0	0	++++	0		0		0		0		0	
30	†	†	†	†	0		0		0		0		0		0	

* All toxic salts showed this pH effect.

† Cells appear to have undergone a change, though not hemolysis.

gested as a working hypothesis, since the impurity was inorganic, that the causative agent was a cation combining with the protein of the cell wall only on the alkaline side of its isoelectric point. That this viewpoint of the mechanism may be entirely erroneous is fully recognized, but as a working hypothesis in the identification of the impurity it proved useful.

It now seemed reasonable to suppose that since all the brands of sodium chloride that contained this impurity came from a natural source, the unpurified or table salt would contain larger quantities of the toxic inorganic substance. Isotonic solutions made from several samples of table salt were found to be turbid because of the ingredients added to prevent caking. On filtering these solutions, it was found

TABLE V

Removal of Impurity by Adsorption on Activated Charcoal and Recovery on Ashing

Salt	1	1N*	2	2N	3	3N	6	6N	1A†	1B‡
Hemolysis Time *min.*										
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	+	0	0	0
4	0	0	++	0	+	0	++	0	0	0
5	0	0	++++	0	++	0	+++	0	S.G.	0
6	0	0		0	+++	0	++++	0	S.G.	0
7	0	0		0	++++	0		0	+	0
10	0	0		0		0		0	++++	0
200	0	0		0		0		0		++++

* N designates salt solution shaken with activated charcoal (Norite).

† NaCl No. 1 plus the ash of charcoal that had been shaken with a toxic salt solution.

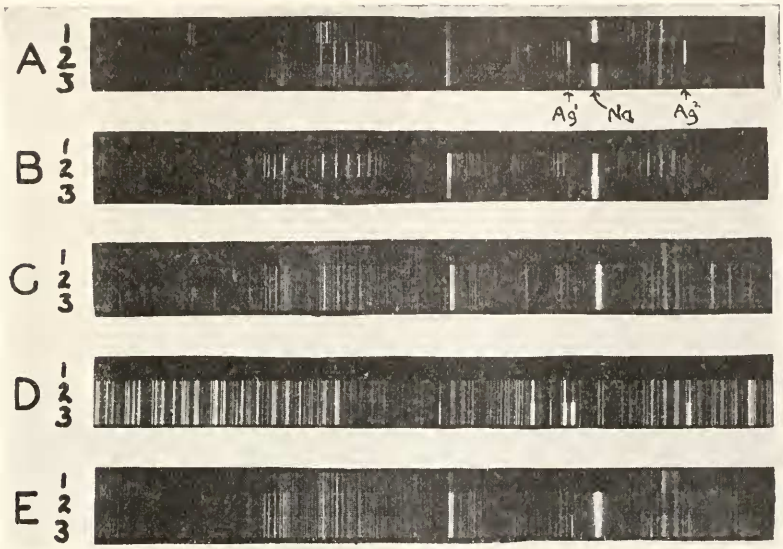
‡ NaCl No. 1 plus the ash of untreated charcoal.

that they caused no hemolysis of fish erythrocytes. A possible explanation for this seemed to be that the toxic principle had been adsorbed and removed by the fine particles that had been filtered off. Though this proved not to be the case, as will be shown later, it suggested attempting to remove the impurity from the toxic solutions by adsorption. Animal charcoal seemed to be the best adsorbent to use, since if the inorganic impurity was adsorbed it could be concentrated by subsequent ashing of the charcoal.

The results given in Table V show that when a toxic solution of sodium chloride is shaken with charcoal and the charcoal removed, the resulting solution has lost its toxic action. If this charcoal is now ashed, the ash treated with HCl, neutralized, and a small portion

added to a non-toxic solution of NaCl, hemolysis occurs. The ash of untreated charcoal also shows a faint hemolytic action though very unlike the pronounced action of the treated charcoal.

Since the adsorption of the impurity on charcoal furnishes a simple



EXPLANATION OF PLATE I

Ag^1—Silver line λ3280.67 A.U.
Ag^2—Silver line λ3382.89 A.U.
Na—Sodium line

A

1. Woods Hole Sea Salt (non-toxic)
2. $AgNO_3$
3. NaCl Brand No. 3 (toxic)

B

1. Graphite Electrodes
2. NaCl Brand No. 4 (non-toxic)
3. NaCl Brand No. 2 (toxic)

C

1. Graphite Electrodes
2. Table Salt No. 1 (non-toxic)
3. Table Salt No. 2 (non-toxic)

D

1. Graphite Electrodes
2. Ash of untreated charcoal
3. Ash of charcoal shaken with NaCl No. 2

E

1. Graphite
2. NaCl No. 3 made non-toxic by shaking with charcoal
3. NaCl No. 3 (toxic)

Obtained with Hilger E1 Quartz Spectrograph, using graphite arc and with technique similar to that described by Shipley, Scott, and Blumberg (1932).

means of purifying sodium chloride solutions for future use, it may be worthwhile to give in detail the procedure followed. To each liter of isotonic salt solution, two grams of animal charcoal (Norite) were added and shaken intermittently throughout the day. The solution

was then allowed to stand overnight and the charcoal filtered off in the morning, the filtrate then being ready for use. The removal of the impurity seems to be a slow process as the addition of more charcoal with more frequent shaking does not enable one to obtain a non-toxic solution in shorter time. It is possible that a small amount of sodium chloride is also removed by the charcoal, which would change slightly the concentration of the solution. This change would be significant only where extremely accurate work is contemplated. Purification of the salt by recrystallization is ineffective.

Since a concentration of the toxic material was now possible by adsorption on charcoal and subsequent ashing, a spectrographic identification of the inorganic impurity seemed possible. Attempts to duplicate the hemolytic action by addition of various inorganic salts to non-toxic solutions had failed.

The results obtained with the spectrograph are shown in Plate I [3] and indicate that silver is the impurity present in the sodium chloride that causes hemolysis of erythrocytes. Those brands of salt that showed hemolysis, Nos. 2 and 3, give the *raies ultimes* of silver. Woods Hole sea water and Brand No. 4, which do not cause hemolysis, contain no detectable silver. It was also impossible to detect silver in two brands of table salt that were non-hemolytic. It had previously been thought that isotonic solutions made from these salts were non-hemolytic due to the removal of the toxic principle by adsorption on insoluble matter contained in them which was filtered off before use.

A comparison of the spectrographs of the ash of charcoal that had been shaken with a toxic salt solution and of the ash of untreated charcoal show the silver lines to be much brighter in the former than in the latter. The presence of silver in the untreated ash explains the delayed hemolysis that resulted on the addition of this ash to a non-toxic salt solution (see Table V). Finally, a comparison of the spectrographs of a toxic sodium chloride before and after shaking with charcoal shows that the silver lines have completely disappeared due to the charcoal treatment. These results would seem to indicate that in whatever state silver exists in the sodium chloride solutions, it is readily adsorbed by activated charcoal, and that the presence of silver is always attended by hemolytic action.

It remained to be shown that hemolysis could be produced by the addition of small amounts of silver to a non-toxic salt solution. Since the marine fish that had been used in the first part of this investigation were not obtainable in Baltimore, it was necessary to use freshwater fish. Several specimens of trout and perch were obtained from the

[3] Run by Mr. Blumberg.

State Hatcheries.[4] The erythrocytes of both these species were hemolyzed rapidly both by a toxic salt solution and by a non-toxic salt solution plus a small amount of silver nitrate. A typical experiment is shown in Table VI.

It can be seen that when silver nitrate is added to a non-toxic salt solution to give a final concentration of added silver equal to 10^{-5} molar, hemolysis occurs in about the same time as in a toxic salt solution. This added silver can then be removed by shaking with charcoal and a non-hemolytic solution obtained. Solutions stronger in silver than 10^{-5} molar are not obtainable, as a precipitate of silver

TABLE VI

Hemolysis of Trout Erythrocytes by Silver

Solution number	1	2	3	4	5
	NaCl No. 2 0.15 M	Solution 1 shaken with charcoal and filtered	Solution 2 plus $AgNO_3$ to give $[Ag] = 10^{-5}$ M	Solution 2 plus $AgNO_3$ to give $[Ag] = 10^{-6}$ M	Solution 3 shaken with charcoal and filtered
Hemolysis Time *min.*					
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	S.G.	0	0
4	S.G.	0	+	0	0
5	++	0	++	0	0
6	+++	0	+++	0	0
7	++++	0	++++	0	0
8		0		0	0
9		0		0	0
10		0		S.G.	0
15		0		+	0
20		0		+++	0
30		0		++++	0

chloride results. Solutions made hemolytic by the addition of silver show the same reaction to pH as a toxic salt solution (see Table IV).

This leaves little doubt that silver is the toxic agent in sodium chloride that causes hemolysis of fish erythrocytes. From a comparison of the rates of hemolysis in a toxic salt solution and a non-toxic salt solution plus known amounts of silver, the concentration of silver in various brands of sodium chloride is found to be of the order of magnitude of 10^{-3} to 10^{-4} per cent by weight.

Other inorganic salts were tested for their hemolytic action. The following salts were added to a non-toxic salt solution to produce concentrations ranging from 10^{-3} to 10^{-6} molar: $AlCl_3$, $AuCl_3$, $BaCl_2$,

[4] Through the courtesy of Mr. Swepson Earle, Commissioner.

$BeCl_2$, $CaCl_2$, $CdCl_2$, $CeCl_4$, $CoCl_3$, $CrCl_3$, CsCl, $CuCl_2$, $FeCl_3$, $HgCl_2$, KCl, $LaCl_3$, LiCl, $MgCl_2$, $MnCl_2$, NH_4Cl, $NiCl_2$, $PbCl_2$, $PdCl_2$, H_2PtCl_6, RbCl, $RhCl_3$, $RuCl_3$, $SbCl_3$, $SnCl_2$, $SrCl_2$, $ThCl_4$, $TiCl_3$, TlCl, $UO_2(NO_3)_2$, VCl_2, $ZnCl_2$. Only mercury and palladium showed marked hemolytic action. Hemolysis by the mercuric salt was always accompanied by a turbidity of the solution. The palladium salt produced a rapid hemolysis giving a clear solution at dilutions similar to that for silver. That palladium is not the impurity present in sodium chloride can be easily shown by the addition of sodium iodide. Concentrations of palladium (10^{-5} to 10^{-6} molar) that cause hemolysis at rates similar to that of toxic salt solution show a pronounced brown coloration on the addition of NaI due to the formation of insoluble palladium iodide. The addition of iodides to a toxic salt solution causes no coloration but does delay hemolysis, probably because AgI is formed, which is more insoluble than AgCl.

The striking similarity in hemolytic action of silver and palladium is of interest for these two elements lie in entirely different groups. Since silver and mercury have been widely used as bactericidal agents [illegible], the possibility suggests itself that palladium might also be useful in this connection, especially since its chloride is so much more soluble than that of silver.

Discussion

In searching for a toxic inorganic impurity in sodium chloride, it is doubtful if silver would be among the first elements to be considered. The low solubility of silver chloride is a well-known fact, and according to the solubility product one would expect its solubility to be still lower in the presence of excess chloride ions. However, there is evidence in the literature to show that silver chloride tends to form soluble complexes in sodium chloride solutions. Pinkus and Berkolaïko (1930) have presented data to show that the solubility of AgCl increases as the concentration of sodium chloride rises. This fact, coupled with the knowledge that quantities of minute importance to the analyst are of extreme physiological significance, tend to lessen the incredibility of the toxic action of silver occurring as an impurity in sodium chloride.

As stated early in this paper, it was found that all brands of salt that showed hemolytic properties were mined. However, crude table salt which is also mined contains no silver, so that it seemed unlikely that the silver occurred in the natural deposits. After much inquiry it was found that the final step in the purification of crude mined salt for the chemical trade was carried out in silver-lined vessels because

less corrosion occurred in such vessels. This then would appear to be the source of the silver that occurs in some brands of sodium chloride.

The presence of silver in sodium chloride as observed here is not a chance occurrence. In a private communication, Dr. E. Wichers states that Mr. A. Isaacs found silver to be present when testing sodium chloride for suitability as a chemical reagent. He also points out that the specifications for sodium chloride published by the American Chemical Society (1928) indicate that silver may be found.

Since presenting this paper before the Society of Biological Chemists, there has come to my attention several instances in which the employment of an artificial biological medium composed of sodium chloride in whole or in part has proved unsuitable. If the brand of sodium chloride used was changed, usually to Kahlbaums, which has been found silver-free, the medium became satisfactory in all respects. That the toxic action of certain brands of sodium chloride is not limited to the hemolysis of fish erythrocytes has been shown by Williams and Jacobs (1931). These workers have found that salts which cause hemolysis usually also exhibit a more toxic action on various other marine organisms. It has also been observed in this laboratory that not only fish but also mammalian erythrocytes undergo hemolysis when placed in isotonic solutions made from sodium chloride containing silver. In the case of mammalian red cells the rate of hemolysis is in terms of hours instead of minutes, though the final effect is none the less definite. Controls showed no hemolysis after several days.

It would, therefore, seem highly appropriate to sound a note of caution in the indiscriminate use of sodium chloride in the preparation of physiological saline solutions, no matter what may be their intended use. Though the effect of the silver, occurring as an impurity, on other biological material may not be as obvious as in the case of fish erythrocytes, it may nevertheless be as serious. Unseen changes in the biological material may have occurred which will alter entirely the response to experimental procedure. The use of silver-free sodium chloride solutions in such work is therefore suggested. Such solutions can easily be prepared by the simple method of purification outlined above.

Summary

1. The hemolysis of fish erythrocytes by isotonic solutions made from certain brands of sodium chloride has been shown with the aid of the spectrograph to be due to silver occurring as an impurity in those salts.

2. The amount of silver present in such salts is of the order of magnitude of 10^{-3} to 10^{-4} per cent and originates from the use of silver-lined vessels in the purification process.

3. A simple procedure for the removal of silver from such salts by adsorption on charcoal has been described.

4. Palladium is the only other element that has been found to be as effective as silver in causing hemolysis.

5. Since the toxic action of silver is not limited to the hemolysis of fish erythrocytes, it is suggested that silver-free sodium chloride be used in the preparation of physiological saline solutions.

BIBLIOGRAPHY

American Chemical Society, Committee on Analytical Reagents, 1928. *Ind. Eng. Chem.*, **20**: 979.

Pinkus, A., and N. Berkolaïko, 1930. *Jour. chim. phys.*, **27**: 364.

Shipley, P. G., T. F. McN. Scott, H. Blumberg, 1932. *Bull. Johns Hopkins Hosp.*, **51**: 327.

Williams, M. M., and M. H. Jacobs, 1931. *Biol. Bull.*, **61**: 485.